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Antioxidant and Thrombolytic Activity of Chloroform Extract of *Bacopa monniera* (L.)

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Abstract

The present study was designed to investigate antioxidant and thrombolytic properties of chloroform extract of *Bacopa monniera* (L.), along with phytochemical study for the presence of phytochemical constituents. Phytochemical analyses were found to be positive for carbohydrates and gums, alkaloids and tannins. Antioxidant potential was evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assays. In DPPH scavenging method, scavenging of DPPH was observed in different concentrations (20, 40, 60, 80, 100, 200, 400, 800µg/ml). Plant extract found to demonstrate significant scavenging activity which was found to increase with concentration of the extract with IC_{50} value of 16.48µg/ml while IC_{50} value of the reference ascorbic acid was 1.61µg/ml. Chloroform extract of *Bacopa monniera* (L.) showed significant ($P < 0.001$) clot lytic properties in different blood samples. The percent clot lytic activity was compared with water (negative control) and standard enzyme streptokinase (positive control). The mean % of clot lysis for water and streptokinase was found 4.70% and 85.77% respectively. On the other hand the mean percent clot lytic activity of chloroform plant extract of *Bacopa monniera* (L.) was found 48.39%, which is significant compare with the positive and negative control.

Keywords: *Bacopa monniera* (L.), DPPH scavenging method, antioxidant potential, streptokinase, clot lytic activity.

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1. Introduction

Different approaches to drug discovery from plants can be enumerated as: random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethnomedical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched/standardized extracts (herbal product development), use of a plant product, biologically potent, as a lead for further chemistry, and single new compounds as drugs (Samuelsson, 1999). The

future of plants as sources of medicinal agents for use in investigation, prevention, and treatment of diseases is very promising (Sofowora, 1982). The objective of the later approach is the targeted isolation of new bioactive plant products, i.e. lead substances with novel structures and novel mechanisms of action. *Bacopa monniera* (L.) (Family: Scrophulariaceae), also referred to as *Bacopa monnieri*, *Herpestis monniera*, water hyssop, and "Brahmi," has been used in the Ayurvedic system of medicine for centuries. Traditionally, it was used as a brain tonic to enhance memory development, learning, and concentration, and to provide relief to patients with anxiety or epileptic disorders. The plant has also been used in India and Pakistan as a cardiac tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction.

Recent research has focused primarily on Bacopa's cognitive-enhancing effects, specifically memory, learning, and concentration, and results support the traditional Ayurvedic claims. Research on anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome, and gastric ulcers also supports the Ayurvedic uses of Bacopa. Bacopa's antioxidant properties may offer protection from free radical damage in cardiovascular disease and certain types of cancer. The present research suggests that the *Bacopa monniera* chloroform extract has moderate thrombolytic activity and significant antioxidant activity. Thus the plant may be a source of effective herbal drug.

2. Materials and Method

Collection and Identification of Plant

The plant *Bacopa monniera* (L.) was collected from University of Chittagong and identified by Syedul Alam (Sohel), Research Assistant, Bangladesh Forest Research Institute, Chittagong. A voucher specimen that contains the identification characteristics of the plant was submitted to the herbarium for future reference.

Preparation of Plant Extract

The fresh *Bacopa monniera* (L.) plant was washed with water immediately after collection. The collected leaves were chopped into small pieces, air dried at room temperature $(25\pm2)^0$C for about 15 days and ground into powder form and stored in an airtight container.200mg powder was macerated in 900 ml pure chloroform for 7 days at room temperature with occasional stirring. 7 days later, chloroform extract was filtered off through a cotton plug and finally with a Whatman No. 1 filter paper. The extract was concentrated under reduced pressure within 50-55^{0}C through rotatory vaccum evaporator (BIBBY STERLIN LTD. ENGLAND). The concentrated extracts were collected in a Petri dish and allow to air dry for complete evaporation of chloroform. The whole process was repeated three times and finally, 23.649 gm blackish-green colored, concentrated stem extract was obtained (yield 16.30 % w/w) which was kept in refrigerator at 4^0C (Ghani, 2003).

Phytochemical Investigation of *Bacopa monniera* (L.)

The freshly prepared crude chloroform extract was qualitatively tested for the presence of chemical constituents. These were identified by characteristic color changes using standard procedures Ghani, 2003), Sofowara (1993), Trease and Evans (1989) and Harborne(1973)

DPPH radical scavenging assay (Brand-Williams *et al*., 1995; Braca *et al.* 2001)

The antioxidant activity of *Bacopa monniera* (L.) chloroform extracts and the standard antioxidant ascorbic acid was assessed on the basis of the radical scavenging effect of the stable 2, 2-diphenyl-1- picrylhydrazyl (DPPH)-free radical activity according to the method described by Brand-Williams *et al*., 1995 with slight modifications. DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can generate stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm (Rice-Evans *et al*., 1997). Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule has an absorbance at 517 nm which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (Brand-Williams *et al*., 1995; Braca *et al.* 2001)).

Experimental Procedure

Bacopa monniera (L.) chloroform extract with different concentrations (20, 40, 60, 80, 100, 200, 400 and 800µg/ml) were prepared in methanol. Ascorbic acid with different concentrations (20, 40, 60, 80, 100, 200, 400 and 800µg/ml) were prepared in methanol. 0.004% DPPH solution was prepared in methanol. 3 ml of this DPPH solution was mixed with 5 ml of extract solution and standard solution separately. These solution mixtures were kept in dark for 30 min. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract (Kumar *et al*., 2008). The absorbance of DPPH solution (Control solution `A') was measured at 517 nm using UV-Visible Spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan). The absorbance of the mixture was determined at 517 nm using UV-Visible Spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan). Ascorbic acid was

served as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity.

The (%) scavenging activity against DPPH was calculated using the following equation:

Scavenging activity (%) = [(A - B) / A] x 100

Where,
A was the absorbance of control (DPPH solution without the sample),
B was the absorbance of DPPH solution in the presence of the sample (extract/ ascorbic acid). Then, % scavenging activity or % inhibition was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis (Hosen *et al*., 2012).

Standard and test solution preparation
Stock solution of plant extract and ascorbic acid made 5mg/ml. Eight screw cap tubes were labeled as 20, 40, 60, 80, 100, 200, 400 and 800µg/ml.5ml of methanol was taken in each screw cap tube.20, 40, 60, 80, 100, 200, 400 and 800µl of methanol were discarded (put off) from the screw cap tubes according to label - using micropipette.20, 40, 60, 80, 100, 200, 400 and 800µl stock solution of conc. 5mg/ml were added (put) accordingly – using micropipette.

***In vitro* thrombolytic activity of *Bacopa monniera* (L.) chloroform extract**
A blood clot (thrombus) developed in the circulatory system due to the failure of homeostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as acute myocardial or cerebral infarction, at times leading to death. Commonly used thrombolytic agents are alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (t-PA) to dissolve clots (Seibert et al., 1994). All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs.

Heparin and Aspirin are only moderately efficient for acceleration of lysis and prevention of reocclusion, but are safe. More selective thrombin inhibitors and anti-platelet agents are more potent, but their safety remains to be confirmed. Continued investigation in this area will provide new insights and promote progress toward the development of the ideal thrombolytic therapy, characterized by maximized stable coronary arterial thrombolysis with minimal bleeding. Several third generation thrombolytic agents have been developed (Tapsell et al ., 2006).
Experimental Procedures:
Streptokinase (SK)
To the commercially available lyophilized SK vial (Durakinase, Dongkook Pharma. Co. Ltd. South Korea) of 15, 00,000 I.U., 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U.) was used for *in vitro* thrombolysis.
Specimen
Whole blood (4 ml) was drawn from healthy human volunteers (n = 10) without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur). 500µl (0.5 ml) of blood was transferred to each of the ten previously weighed microcentrifuge tubes to form clots.
Herbal preparation
100 mg *Bacopa monniera* (L.) Chloroform extract was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22-micron syringe filter. 100µl of this aqueous preparation of herb was added to the microcentrifuge tubes containing the clots to check thrombolytic activity.
Clot lysis
4 ml venous blood drawn from healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5ml/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To one micro centrifuge tube containing pre-weighed clot, 100µl of aqueous extract of *Bacopa monniera* (L.) swas added. As a positive control, 100µl of SK and as a negative non thrombolytic control, 100µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37^0C for 90 minutes and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated 10 times with the blood samples of 10 volunteers.

3. Results and Discussion

Phytochemical Screening (Zhang and Guo, 2001; Zhao *et al*.; 2003 and Zhao *et al*., 1992)

Phytochemical screening of *Bacopa monniera* (L.) extract under this study explored the presence of medicinally active secondary metabolites carbohydrates and gum, alkaloid, and tannins. This investigation also indicated the absence of reducing sugar, steroid, glycoside and flavonoid. These findings with their corresponding results are summarized in **Table 1**.

DPPH radical scavenging assay

DPPH free radical scavenging method was used for the assay of *Bacopa monniera* (L.) chloroform extract and the scavenging activity was compared with the standard antioxidant ascorbic acid (Vitamin C). The DPPH free radical scavenging activity of the *Bacopa monniera* (L.) chloroform extract and ascorbic acid is shown in **Table 2 and Table 3 Fig. 1, 2**. Both ascorbic acid and *Bacopa monniera* (L.) chloroform extract showed dose dependent activity. Among the eight different concentrations used in the study (20, 40, 60, 80, 100, 200, 400 and 800µg/ml) ascorbic acid showed 67.78%, 72.89%, 77.82%, 82.75%, 87.15%, 89.26%, 93.13% and 95.95% scavenging activity where highest scavenging activity was 95.95% at concentration 800µg/ml (**Table 2**). On the other hand, *Bacopa monniera* (L.) extract showed 44.89%, 64.61%, 65.14%, 66.20%, 80.81%, 81.34%, 82.92% and 93.66% scavenging activity at the above mentioned eight different concentrations where highest scavenging activity of *Bacopa monniera* (L.) chloroform extract was 93.66% at concentration 800µg/ml (**Table 3**).

% of scavenging activity or % of inhibition was plotted against log concentration and from the graph IC_{50} (Inhibition concentration 50) value was calculated by linear regression analysis. IC_{50} value of ascorbic acid and *Bacopa monniera* (L.) chloroform extract was found 1.61 and 16.48µg/ml respectively (**Table 2 and Fig 1, 2 & 3**).

***In vitro* thrombolytic activity of *Bacopa monniera* (L.) chloroform extract**

Addition of 100µl Streptokinase (Durakinase, Dongkook Phama. Co. Ltd, South Korea), a positive control (30,000I.U.) to the clots along with 90 minutes incubation at 37^0C, showed 85.77% clot lysis. On the other hand, clots when treated with 100µl sterile distilled water (negative control) showed only negligible clot lysis which was only 4.70%. The mean difference in clot lysis percentage between positive and negative control was very significant (***p value < 0.001). But when 100µl *Bacopa monniera* (L.) chloroform extract was added to 10 different clots, 48.39% clot lysis were obtained and when compared with the negative control(water) the mean clot lysis percentage differences was significant (***p value < 0.001). Percent clot lysis obtained after treating with water, streptokinase and *Bacopa monniera* (L.) chloroform extract shown in **Fig.4**. Statistical representation (*Student's t-test*) of the effective clot lysis percentage by *Bacopa monniera* (L.) chloroform extract, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) is tabulated in **Table 4.** Percentage of clot lysis of 10 different blood samples after treated with water, streptokinase and *Bacopa monniera* (L.) chloroform extract is shown in Table5 Here, Clot weight = weight of clot containing tube – weight of tube alone. All the tubes were incubated at 37^0C for 90 minutes and observe for clot lysis. After Incubation fluids released was removed and tubes were again weighted to observe the difference in weight after clot disruption. Differences obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was carried out with blood sample of 10 volunteer.

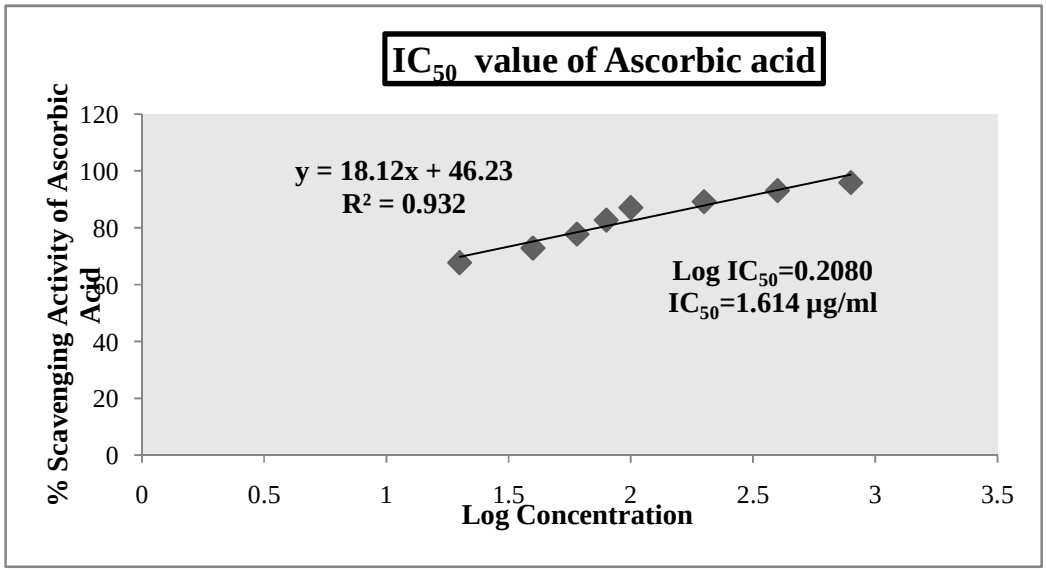

Figure 1. IC_{50} value of ascorbic acid was calculated from above plot linear regression analysis.

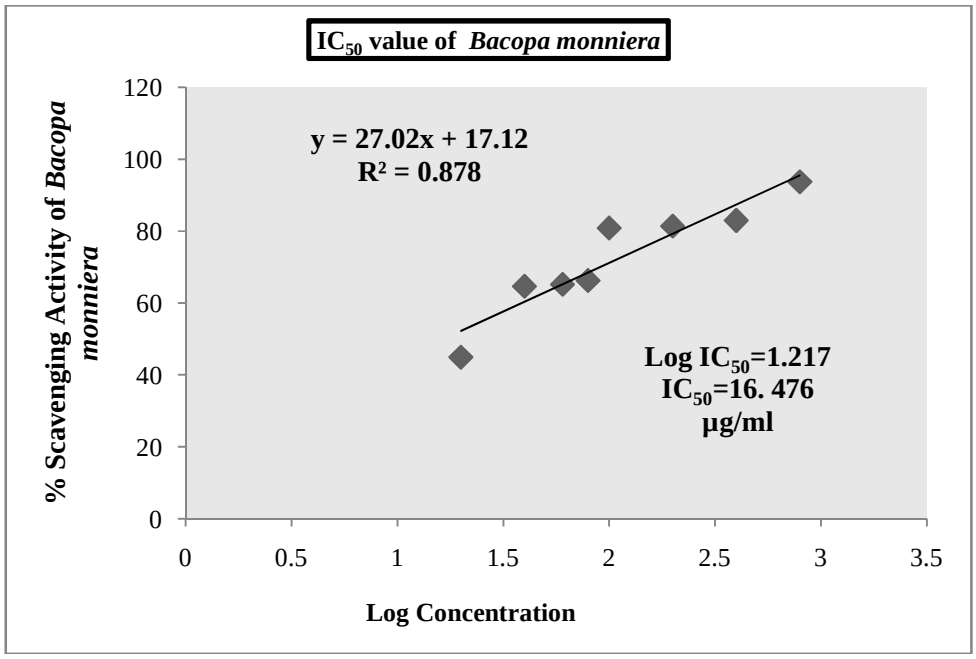

Figure 2. IC_{50} value of *Bacopa monniera* (L.) chloroform extract was calculated from above plot linear regression analysis

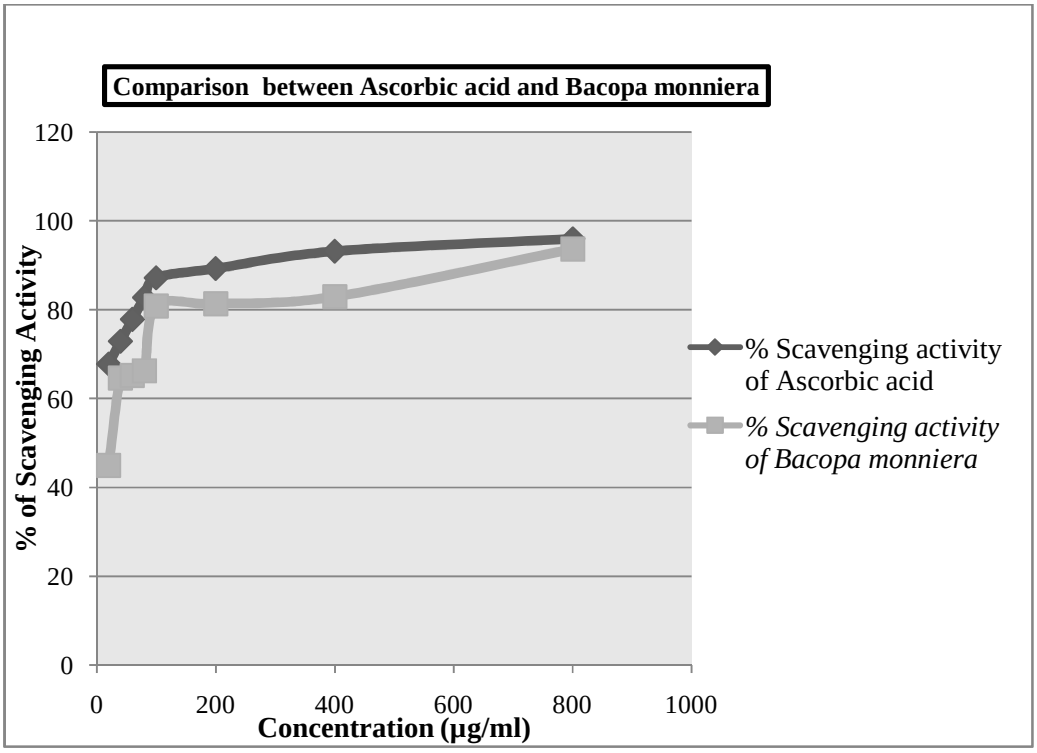

Figure 3. Relative % scavenging activity or % inhibition of standard antioxidant ascorbic acid and *Bacopa monniera* (L.) Chloroform extract

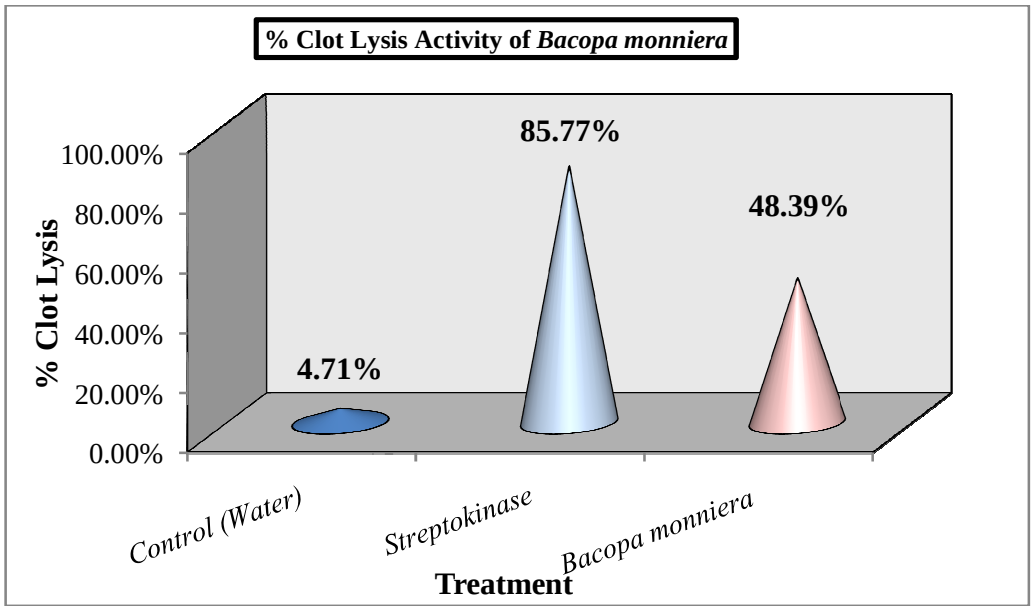

Figure 4. Comparative % in vitro thrombolytic effect of *Bacopa monniera* (L.) chloroform extract, streptokinase and water (negative control)

Table1: Observation and Result of Phytochemical screening

Secondary metabolite	Name of the test	Observation	Result
Carbohydrate and Gums	Molish test	Red violet ring was produced	++
Reducing sugar	Fehling's solution test	No Brick red color ppt.	--
	Benidict's test	No Red color ppt.	--
Alkaloids	Dragendroff's test	Orange brown ppt.	++
	Wagner's test	No Reddish brown ppt.	--
	Hager's test	No Yellowish ppt.	--
	Mayer's test	Yellow color ppt.	++
Steroids	Salkowski reaction	No Red color in chloroform layer	--
	Libermann-Burchard reaction	No Light green color	--
Glycosides	Salkowski reaction	No Red color in chloroform layer	--
	Libermann-Burchard reaction	No Light green color	--
Tannins	Ferric chloride test	No black ppt. was present	--
	Potassium dichromate test	Orange ppt.	++
Flavonoids	Hydrochloric acid test	No red color	--
Saponins	Foam test	No foam production	--

N.B. "++" stands for the presence and "--" indicates the absence of secondary metabolites.

Table2: DPPH free radical scavenging activity of ascorbic acid (Standard)

Ascorbic acid				**IC_{50}**
Concentration (µg/ml)	**Log Concentration**	**Absorbance**	**% Scavenging activity**	
Control	-	0.568	-	
20	1.30	0.183	67.78	
40	1.60	0.154	72.89	
60	1.78	0.126	77.82	**1.61µg/ml**
80	1.90	0.098	82.75	
100	2.00	0.073	87.15	
200	2.30	0.061	89.26	
400	2.60	0.039	93.13	
800	2.90	0.023	95.95	

Table 3: DPPH free radical scavenging activity of *Bacopa monniera* (L.) chloroform extract

***Bacopa monniera* (L.) chloroform extract**				**IC_{50}**
Concentration (µg/ml)	**Log concentration**	**Absorbance**	**% Scavenging activity**	
Control	-	0.568	-	
20	1.30	0.313	44.89	
40	1.60	0.201	64.61	
60	1.78	0.198	65.14	**16.48 µg/ml**
80	1.90	0.192	66.20	
100	2.00	0.109	80.81	
200	2.30	0.106	81.34	
400	2.60	0.097	82.92	
800	2.90	0.036	93.66	

Table 4: Effect of *Bacopa monniera* (L.) chloroform extract on *in vitro* thrombolysis

Herbal/ Drug	**% Clot Lysis**
Control (water)	4.70
Streptokinase	85.77
Bacopa monniera (L.) chloroform extract	48.39

Table5: Comparing the data of % of Clot lysis using SPSS 11.5
Group Statistics (Control vs. Streptokinase)
Group Statistics

	Control, Streptokinase	N	Mean	Std. Deviation	Std. Error Mean
% of Clot lysis	Control	10	4.7990	.65113	.20590
	Streptokinase	10	85.7720	1.12211	.35484

Independent Samples Test

			% of Clot lysis	
			Equal variances assumed	Equal variances not assumed
Levene's Test for Equality of Variances	F		5.950	
	Sig.		.025	
t-test for Equality of Means	T		-197.372	-197.372
	Df		18	14.444
	Sig. (2-tailed)		.000	.000
	Mean Difference		-80.9730	-80.9730
	Std. Error Difference		.41026	.41026
	95% Confidence Interval of the Difference	Lower	-81.83491	-81.85038
		Upper	-80.11109	-80.09562

Group Statistics

	Control, Streptokinase	N	Mean	Std. Deviation	Std. Error Mean
% of Clot lysis	Control	10	4.7990	.65113	.20590
	Bacopa monniera	10	48.3860	4.62638	1.46299

Group Statistics (Control vs. *Bacopa monniera*)

			% of Clot lysis	
			Equal variances assumed	Equal variances not assumed
Levene's Test for Equality of Variances	F		18.882	
	Sig.		.000	
t-test for Equality of Means	T		-29.502	-29.502
	Df		18	9.356
	Sig. (2-tailed)		.000	.000
	Mean Difference		-43.5870	-43.5870
	Std. Error Difference		1.47741	1.47741
	95% Confidence Interval of the Difference	Lower	-46.69092	-46.90982
		Upper	-40.48308	-40.26418

Independent Samples Test
Group Statistics (*Bacopa monniera* (L.) Vs. Streptokinase)
Group Statistics

	Control, Streptokinase	N	Mean	Std. Deviation	Std. Error Mean
% of Clot lysis	Streptokinase	10	85.7720	1.12211	.35484
	Bacopa monniera	10	48.3860	4.62638	1.46299

Independent Samples Test

			% of Clot lysis	
			Equal variances assumed	**Equal variances not assumed**
Levene's Test for Equality of Variances	F		14.049	
	Sig.		.001	
t-test for Equality of Means	T		24.834	24.834
	Df		18	10.055
	Sig. (2-tailed)		.000	.000
	Mean Difference		37.3860	37.3860
	Std. Error Difference		1.50541	1.50541
	95% Confidence Interval of the Difference	Lower	34.22326	34.03424
		Upper	40.54874	40.73776

Here, all values are expressed as MEAN ± SEM (n=20)
*****P<0.001 significant compared to negative control**

4. Conclusion

A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs" (Sofowora, 1982). Medicinal plants constitute an important natural wealth of a country. They play a significant role in providing primary health care services to rural people. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine. In the present study, studied on chemical properties was carried out on *Bacopa monniera* (L.) (Family: Scrophulariaceae). Traditionally, it was used as a brain tonic to enhance memory development, learning, and concentration, and to provide relief to patients with anxiety or epileptic disorders. The plant has also been used in India and Pakistan as a cardiac tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction. Chemical group tests were analyzed qualitatively on *Bacopa monniera* (L.) and it showed that carbohydrates and gum, alkaloid, and tannins were present in the chloroform extract of *Bacopa monniera* (L.) The thrombolytic study shows that, it has significant thrombolytic activity. The antioxidant study shows that it has significant antioxidant activity.

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